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14. ABSTRACT

Objective of this application was to define the regulation of heparanase (HPSE) leading to a modulation of brain-metastatic melanoma (BMM). This goal was to be achieved by employing: a) novel HPSE inhibitors that either we have discovered, e.g., miR-1258, a microRNA targeting human HPSE and suppressing its expression, or have been recently developed, e.g., SST0001, a small molecule, membrane-permeable, nonanticoagulant glycol - split heparin and potent inhibitor of HPSE activity; b) a human BMM model recapitulating the stages of melanoma progression to the BMM phenotype; c) pINDUCER as novel inducible shRNA/cDNA expression lentiviral system which enables tracking of viral transduction and the uniform control of heparanase in cells and xenografts, thus minimizing experimental variations due to tumor heterogeneity.

15. SUBJECT TERMS
Brain-metastatic Melanoma (BMM), MicroRNA, Heparanase (HPSE)

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1. INTRODUCTION:

Patients with brain metastatic melanoma (BMM) have, even with the best available treatment, a median survival of less than six months. Unfortunately, mechanisms underlying BMM remain largely unknown. The key to improve treatment of patients with BMM lies in developing better mechanistic understandings of this disease in order to successfully block melanoma dissemination to the brain. The long-term objectives of Marchetti's laboratory are to determine the molecular determinants of brain metastasis, how it is regulated, and to use this knowledge to develop more effective therapies combating brain metastasis. The current study was designed to provide new and important discoveries deciphering mechanisms of BMM disease.

2. KEYWORDS:

KEYWORDS: Brain-metastatic melanoma (BMM), MicroRNA-1258, Heparanase (HPSE).

3. OVERALL PROJECT SUMMARY:

This report represents the progress made during the first year of the DoD-CDMRP Discovery Award. We have addressed sub-tasks of the original specific aim 1 of the proposal. The overall purpose of this aim was to test efficacies of the heparanase (HPSE) target microRNA, microRNA1258, suppressing BMM in tumor xenografts, either alone or in combination with the HPSE inhibitor SST0001. The objective was also to identify abilities of *heparanase*-targeting MicroRNA-1258 (miR-1258) to suppress brain metastatic melanoma (BMM). The sub-tasks of aim 1 of the original Statement of Work (SOW) have been highlighted below, together with responses of progress made, in bold:

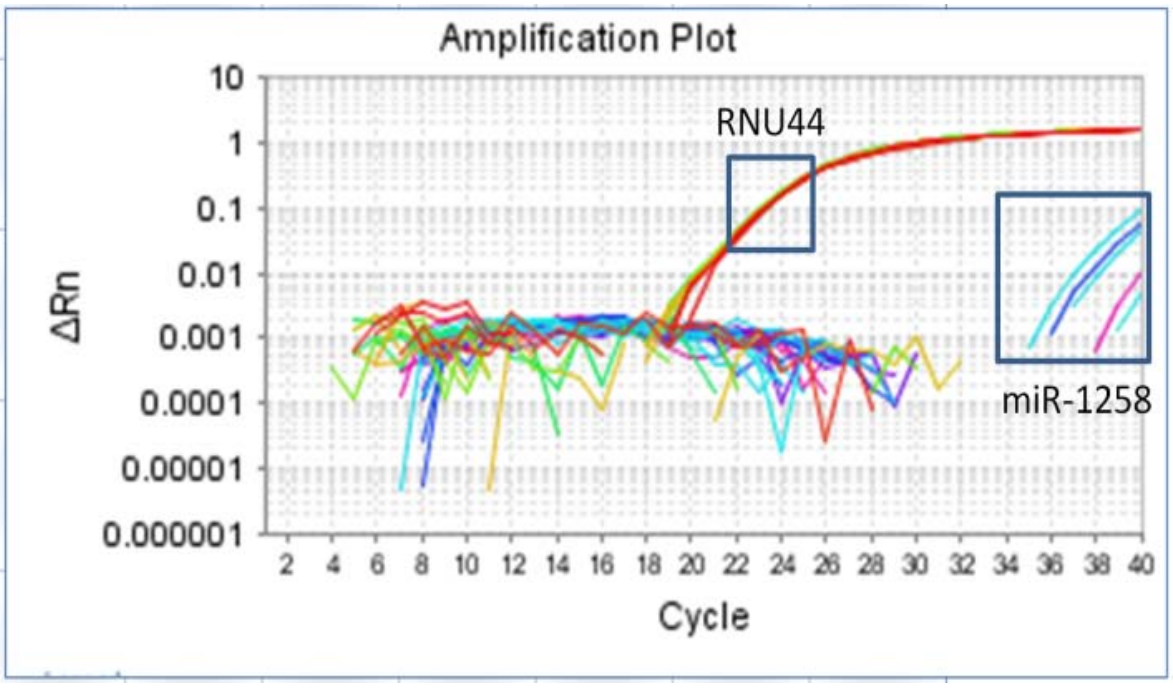
1. An initial period is requested for ACURO approval (1-3 months).
   This period was employed to receive ACURO approval.

1a. Transduce human BMM cell lines (SM4, 70W, SB1B) and parental non-metastatic melanoma counterparts (MeWo, SBCl3) with the microRNA-1258 (miR-1258) lentivirus and corresponding controls (Dharmacon, Inc.). Determine the regulation of heparanase (HPSE) expression, activity, and heparanase subcellular localization by confocal microscopy (months 1-4).

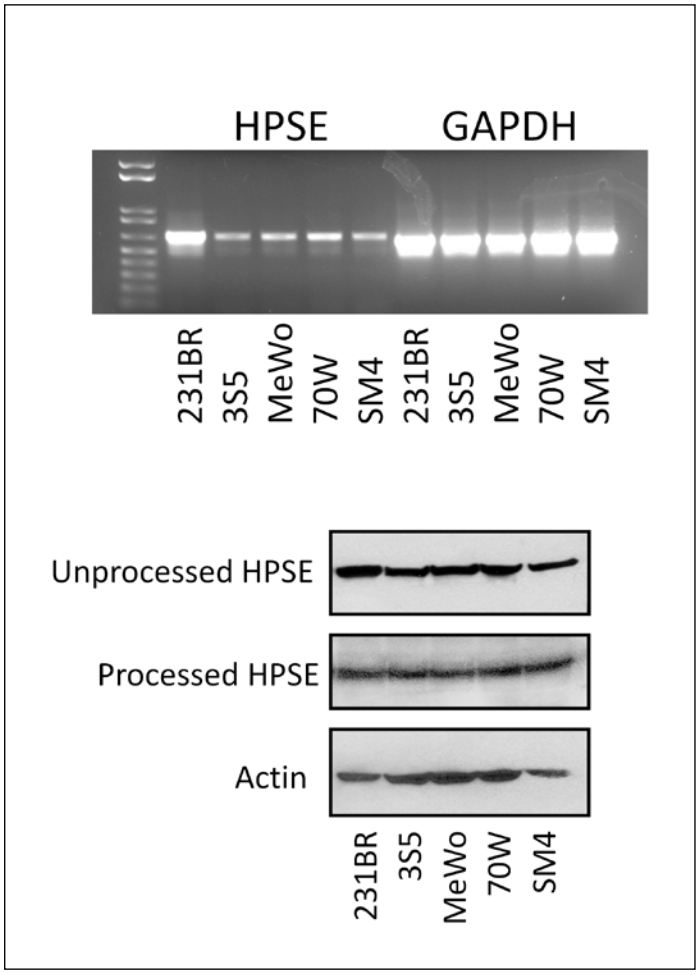
1b. Perform real-time polymerase chain reaction (PCR), Western blot analyses, and HPSE

activity assays in BMM cells to assess HPSE transcripts, proteins, and activity levels regulated by miR-1258 and SST0001 in BMM cells (months 1-4).

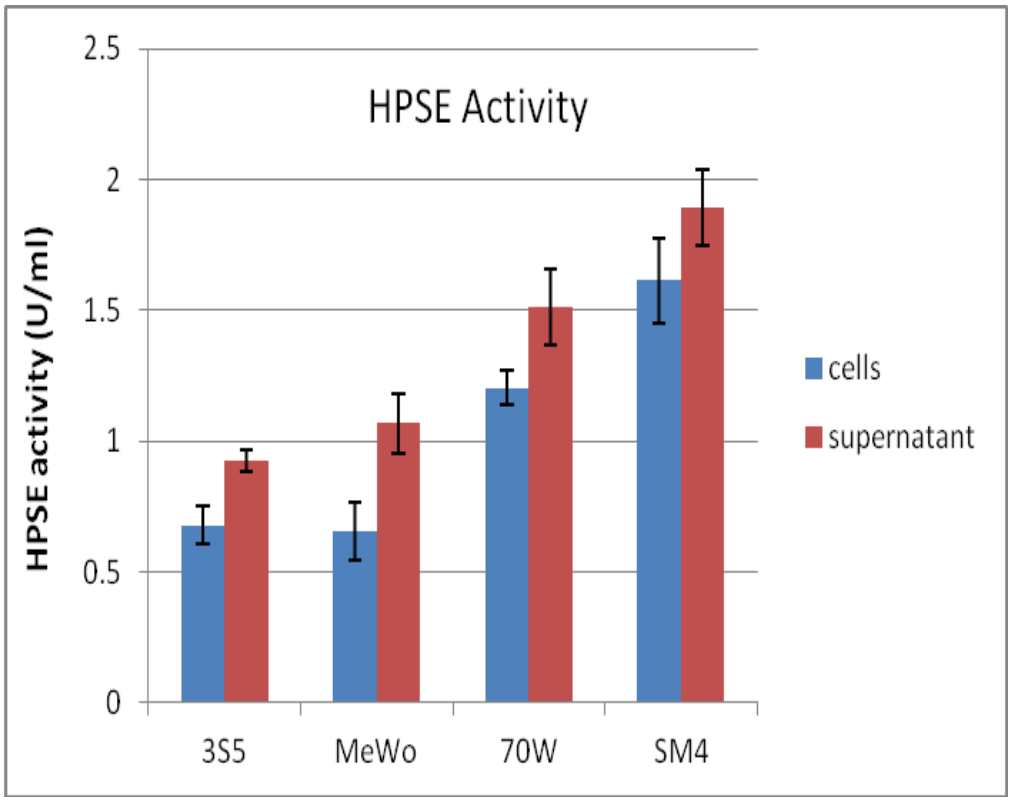
We hypothesized that miR-1258 levels would be different among cell lines (3S5, MeWo, 70W and SM4). However, levels of this miRNA could not be detected by real-time PCR (RT-PCR) while controls for RT-PCR (e.g., the use of RNU44) showed expression in all cell lines. A representative example of analyses employing real-time PCR is shown in the figure below. Levels of miR-1258 in the melanoma cell lines are indicated by color (e.g., blue, light blue, and pink lines) were not detectable by real-time PCR in any of the cell lines (C_T is above cycle 36), while control RNU44 (colored lines of the figure insert) showed expression in all melanoma cell lines (C_T values at cycle 23). Real-time PCR experiments were performed multiple times, using different batches of cells, and at different stages of cell growth. We concluded that this specific microRNA is not detectable in human melanoma cell lines, and is not naturally expressed in melanoma cells. This outcome altered this and the other sub-tasks of aim 1.



We also hypothesized that a correlation between heparanase levels and BMM propensities would be detected in the human melanoma cell systems considered. To this end, we performed mRNA (by RT-PCR) and protein (by Western blotting) analyses to investigate HPSE expression at the mRNA and protein levels. We could not observe any specific differences in HPSE transcript or protein expression among the human melanoma cell lines considered (figure below. Top panel represents RT-PCR, bottom panel represents Western blotting analyses). Of note, HPSE protein differences could not be detectable either using the latent, un-processed form of HPSE (62.5 kDa) by using a specific antibody to it, nor the processed form of HPSE (50 kDa) employing a specific antibody developed against this form of the molecule. The figure shown below is representative of multiple RT-PCR and Western blotting analyses that were performed on these cells using the brain-metastasizing human 231BR breast cancer variant as positive control for HPSE transcript and protein expression.



Conversely, we detected increased levels of heparanase (HPSE) activity in human melanoma cell lines according to their brain-metastatic propensities. Specific HPSE activity was measured by the heparan degrading enzyme assay kit (Takara Mirus Biomedical, Takara , Japan). Importantly, we could detect HPSE activity in both cell lysates and culture media from human melanoma cells. Furthermore, we observed a significant correlation between HPSE activity and BMM propensities in either cell lysates or supernatants (e.g., secreted HPSE), with highest levels detected in the BMM 70W series, parental 70W and the super-BMM 70WSM4 variant. Data are displayed in Figure 3, shown below, and are representative of four independent investigations.



1c. Perform cell adhesion, migration, and invasion assays (chemoinvasion/brain slice/BBB transmigration models) in BMM cells transduced with miR-1258 lentivirus or antisense control, and/or treated with SST0001 in a dose- and time-dependent manner (months 2-5).

This sub-task could not be achieved because of the negative results obtained by real – time

PCR and indicating an absence of specific miR-1258 signal in human melanoma cell lines.

1d. Inject miR-1258 lentiviral-transduced BMM cells in animals, and carry-out experimental metastasis assays to demonstrate the *in vivo* physiological relevance of SST0001 in the presence or absence of miR-1258 lentiviral transduction.

This sub-task could not be achieved because of the negative results for miR-1258 analyses.

1e. Detect, enumerate, and compare micro- and macro-metastatic BMM lesions and HPSE expression in mice populations injected with BMM cells transduced with miR1258 and/or treated with optimized doses of SST0001. Preparation and submittal of a manuscript for dissemination of findings in a peer-reviewed journal (month 9-12).

This sub-task tasks could not be achieved because of the negative results obtained by miR-1258 analyses.

4. KEY RESEARCH ACCOMPLISHMENTS

- Results show that microRNA-1258 is not naturally expressed in human melanoma cell lines considered in this study. This contrasts to microRNA-1258 detection in human breast cancer cell systems and suggests that a microRNA-dependent regulation of HPSE can be cancer type-specific. Therefore, findings achieved in one cancer type do not translate to another, notably, microRNA-dependent HPSE mechanisms in human melanoma.
- We have demonstrated a differential presence of HPSE activity in human melanoma cells compared to their supernatants, and according to brain metastatic potential of these cells.

5. CONCLUSION

By implementing the proposed sub-tasks, experimental efforts resulted in conflicting outcomes affecting the project performance for aim 1. Results indicate that microRNA-1258 is not naturally present in human melanoma cell lines considered in this study. This contrasts to microRNA detection in human breast cancer cell systems and suggests that the

microRNA regulation of HPSE can be cancer type-specific. Therefore, findings achieved in one cancer type do not translate to another, notably, melanoma. Secondly, we have demonstrated a differential presence of HPSE activity in human melanoma cells compared to their supernatants. In addition, and of interest, we found that both HPSE activities increased according to the brain metastatic potential of these cells. This can be of relevance for the implementation of studies using HPSE inhibitors like the heparinoid SST0001 or other HPSE inhibitors that have been developed and have become available.

6. PUBLICATIONS, ABSTRACT, AND PRESENTATION

None in the specific melanoma area that was investigated.

7. INVENTIONS, PATENTS AND LICENSES:

None.

8. REPORTABLE OUTCOMES

None.

9. OTHER ACHIEVEMENTS

None.

10. REFERENCES

No new references.

11. APPENDICES:

None.

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